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# Mass Spectrometric Structure Determination of Spider Toxins: Arginine-Containing Acylpolyamines From Venoms of Brazilian Garden Spider *Nephilengys cruentata*

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**ABSTRACT** A new strategy to characterize glutaminergic blocker acylpolyamines stored in a spider venom with mass spectrometry is described. The crude spider venom extracts are amenable to direct MALDI mass spectrometry analysis which provides a rapid and accurate means of measuring the molecular weights of acylpolyamines without the isolation of individual samples. Compared with the previously developed μ-column HPLC/MS method, this procedure provides more efficient detection and identification of complex venom constituents. Twenty-five acylpolyamines were detected from Brazilian garden spider *Nephilengys cruentata* crude venom extracts by both HPLC/MS and MALDI-mass spectrometry. These acylpolyamine structures were determined by high-energy collision induced dissociation MS/MS method. Most of the compounds were classified into the previously reported generalized structures types A to D, which were found from the venom of *Nephilengys borbonica.* The structures of four acylpolyamines $(M + H)^+$, m/z 623, 646, 688, and 745, which were not contained in the venom of *Nephilengys borbonicare* were determined to have arginine at the polyamine chain terminal and were named NPTX-622, -645, -687, and -744, respectively. *Nat. Toxins 5:47–57, 1997.* 

**Key Words:** spider venom; acylpolyamines; matrix-assisted laser desorption ionization (MALDI)-mass spectrometry; high-energy collision induced dissociation (CID)

## INTRODUCTION

Venomous animals store various complex substances which often exhibit specific biological activities, and some of them have been used as tools in neurochemical research. But full characterization of the venom constituents was still difficult because of structurally related complex mixtures [McCormick and Meinwald, 1993]. Recently, with the progress of isolation and bioassay methods, spiders are receiving high attention in the search for new neurotoxic substances and have yielded a series of low molecular weight glutaminergic blocker acylpolyamines [Aramaki et al., 1987a,b; Adams et al., 1987; Toki et al., 1988; Budd et al., 1988; Grishin et al., 1989; Jasys et al., 1990; Quistad et al., 1990; Chiba et al., 1994,1995]. We have also reported a highly sensitive analytical method to characterize complex neurotoxic compounds extracted from spider venoms using fast-atom bombardment (FAB) and four-sector tandem mass spectrometry (MS/MS) [Nakajima and Itagaki, 1996; Itagaki et al., 1996]. With the use of μ-column HPLC/FAB-MS system, rapid identification of nearly every acylpolyamine in a crude venom extract is permitted. From the crude venom extracts of *Nephilengys borbonica*, it was possible to identify 40 acylpolyamines, and among those only five compounds were previously known [Itagaki et al., 1996]. When compared with the traditional procedures for elucidating the structures of spider toxins which have been the use of high performance liquid chromatography to collect biologically active fractions, followed by proton nuclear magnetic resonance measurements and/or hydrolysis to amino acids and amines, this μ-column HPLC/FAB-MS system can be seen as an advanced method because all venom constituents regardless of being major or minor substances can be detected at the same time with high analytical accuracy and less sample. But still, the use of μ-column LC requires time and laborious efforts to obtain analytical results. Matrix-assisted laser desorption ionization (MALDI) mass spectrometry has emerged as an important technique for the analysis of biological complex mixtures without prior isolation

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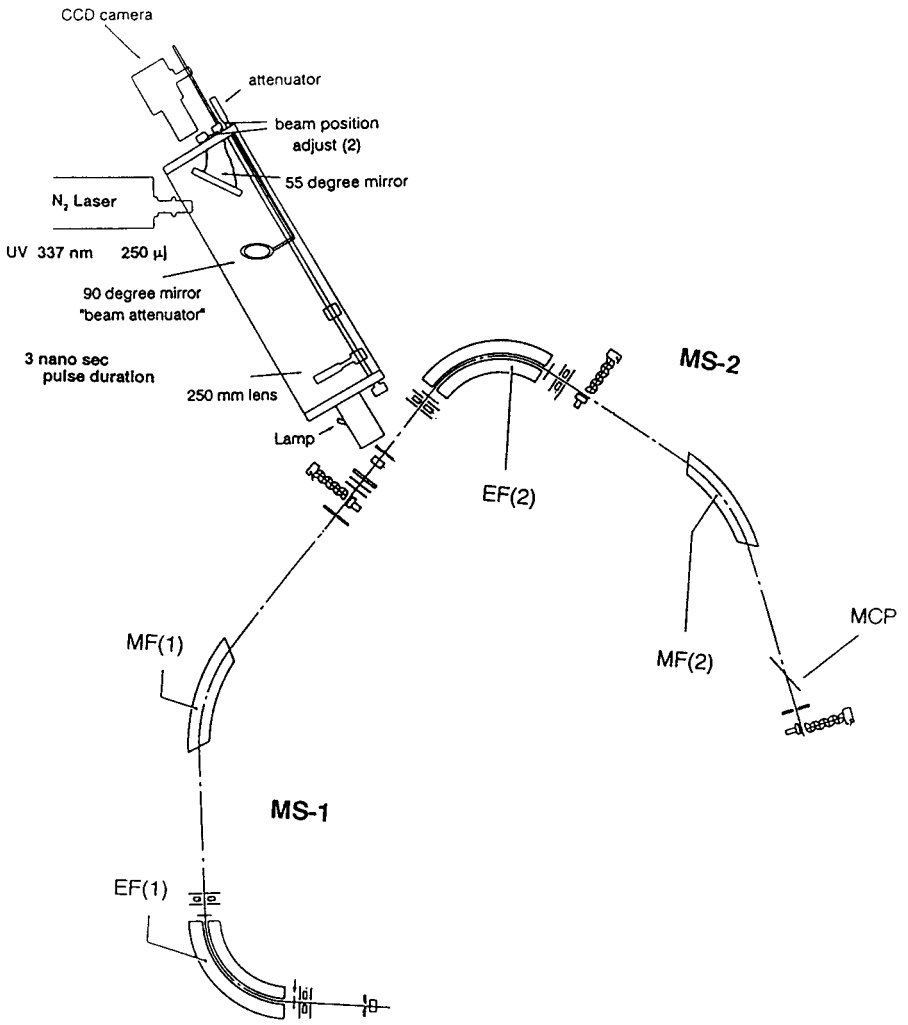


**Fig. 1.** Schematic diagram of tandem mass spectrometer equipped with MALDI source at MS-2 Ion source.

procedures [Billeci and Stults, 1993; Cain et al., 1994; Szilágyi et al., 1996], but the low resolving power of commercial linear MALDI-time-of-flight (TOF) mass spectrometers has restrict the application of MALDI in the low mass region. Since the introduction of reflector type TOF, delayed ion source equipped TOF or combination of MALDI with sector MS [Mamyrin, 1994; Vestal et al., 1995; Annan et al., 1992; Bordoli et al., 1994], high-resolution conditions can be set easily and the studies of low molecular weight [i.e., below 1,000 Dalton (Da)] compounds was attained avoiding overlap with the complex matrix signals.

This paper describes the identification of arginine containing NPTX-622, -645, -687, and -744 acylpolyamines and also the classification of the remaining acylpolyamines possessing structures common to those determined previously in *Nephilengys borbonica* venom, into the previously reported structure type-A to -D. Both HPLC/MS and MALDI-MS methods were applied for the analysis of the crude venom extracts of *Nephila cruentata* and confirmed the utility of MALDI-MS for direct mixture analysis.

## MATERIALS AND METHODS

### Collection of Spider Venom and Extraction

Spiders (*Nephilengys cruentata*) were collected from a wooded area in Rio Claro, SP Southeast of Brazil. Venom glands were taken out and kept in a deep freezer (at −80°C). Each venom gland (1.20 mg) was homogenized in 2 ml of 60% acetonitrile-aqueous solution (containing 0.1% trifluoroacetic acid [TFA]) and was centrifuged at 14,000 rpm/min for 1 h. The supernatant fluid was filtered through Microcon 3 (Amicon, Beverly, MA) to eliminate materials over 3,000 Da. The filtrate was diluted to 1/10th the original concentration with water and was used for the LC/MS, MS/MS, and MALDI-MS measurements without any further treatment. Following previously reported extraction method [Toki et al., 1988], the supernatant fluid was also filtered through TOSOH-Sep-Pack cartridge (C18 plus) with 30% acetonitrile-aqueous solution (0.05% TFA) to obtain the acylpolyamine fraction. The acylpolyamine fraction was analyzed by reverse-phase TSK-GEL ODS-80TM column chromatogra-

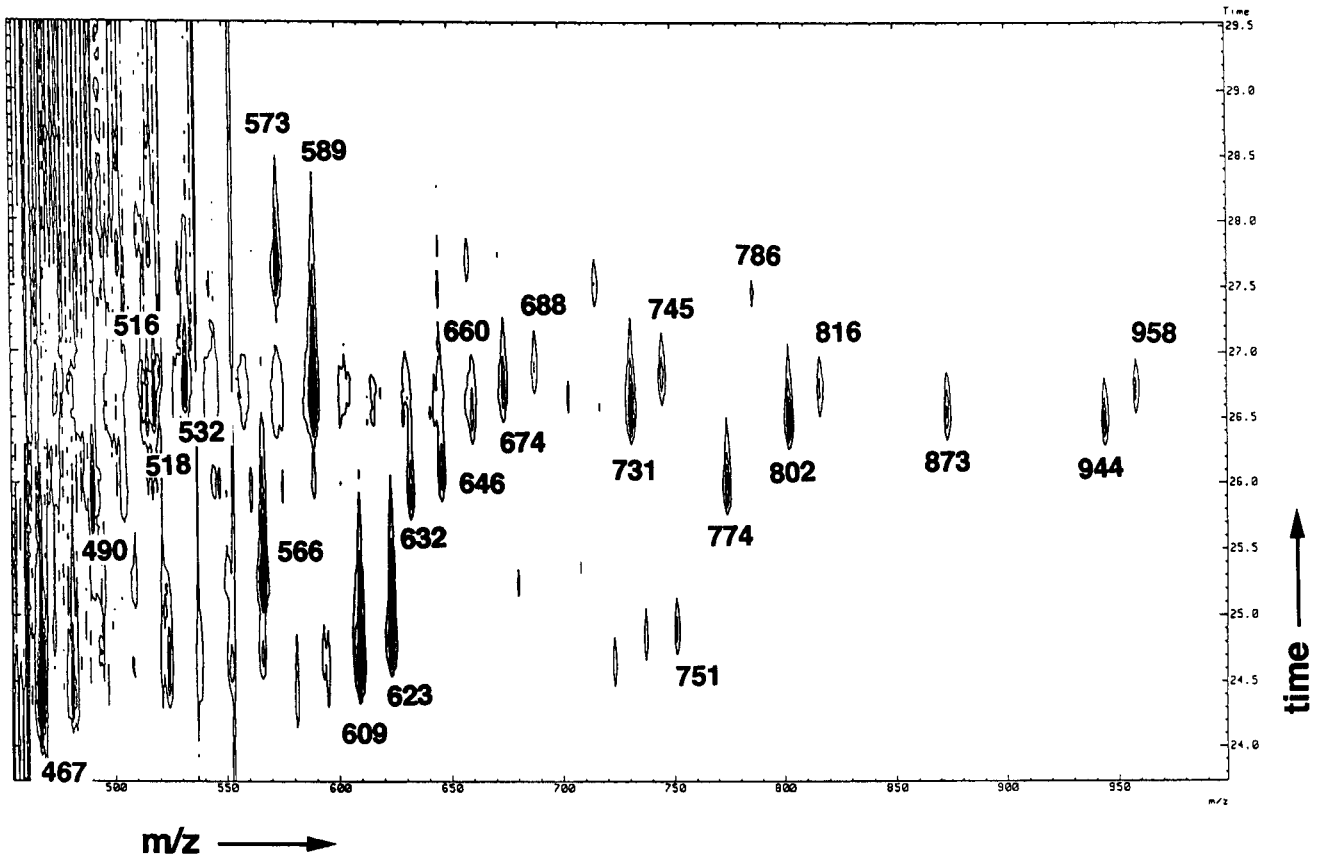


**Fig. 2.** 2-Dimensional mass chromatogram display of protonated molecular ions $(M + H)^+$ obtained from *Nephilengys cruenta* venom extracts using on-line μ-column HPLC-FAB mass spectrometer.

phy as described by [Toki et al., 1988] and yielded three acylpolyamine sub-fractions for NMR measurements.

### LC/MS System

A detailed description of this interface is described elsewhere [Itagaki et al., 1996] and the same operational conditions were used for the experiment. Two LC110P model pumps (Yokogawa, Tokyo, Japan) delivered solvents to a gradient solvent mixer (10 μl). After mixing the solvents, the gradient flow was split into 200:1 using a restriction column prior to introduction to the valve injector. The sample, injected through a conventional Reodyne sample injector (Model 7125) equipped with a 10 μl injection loop, flowed onto a 0.3 × 150 mm Develosil ODS-HG-5 μ-column (Nomura Chemical, Seto-shi, Japan). Samples were concentrated at the head of the μ-column by the isocratic use of water. Components eluted under gradient conditions, flowed through a 60 μm (i.d.) fused silica capillary again and mixing at the Valco ZT.5(1/32)T with the matrix solution. The mixture was pumped out by a syringe pump Model 22 (Harvard Apparatus, South Natick, MA) and was injected into a JEOL FRIT-FAB interface at a flow rate of 4 μl/min. Solvents A ($H_2O$/TFA = 100/0.1 [v/v]) and B ($CH_3CN$/TFA = 100/0.1 [v/v]) were prepared and used for LC/MS. As a standard procedure, solvent A was kept isocratic for 5 min, then a linear gradient of solvent B (0–80%) was applied over 20 min.

### Mass Spectrometry

#### MALDI-Mass Spectrometry

MALDI-Sector mass spectrometry experiments were performed on the MS-2 of a conventional JEOL HX110A/110A four sector tandem mass spectrometer equipped with a MALDI unit (JEOL USA, Peabody, MA) and a MS-ADS11 variable mass dispersion array detector at MS-2 (Fig. 1). Nitrogen laser 337 nm with 3 nsec pulse duration was used for the ionization. Acceleration voltage was 10 kV and resolution was set around 1,000. All samples used for MALDI were dissolved in a saturated aqueous solution of 2,5-dihydroxybenzoic acid (DHB). Then 5 μl of the resulting solution was applied to the gold-plated tip of a FAB probe and either air- or vacuum-dried. An array detector was set to cover the mass range 1:1.1.

MALDI-TOF spectra were measured on delayed ion source and collision cell equipped Voyager Elite TOF mass spectrometer (PerSeptive Biosystem, Framingham, MA).

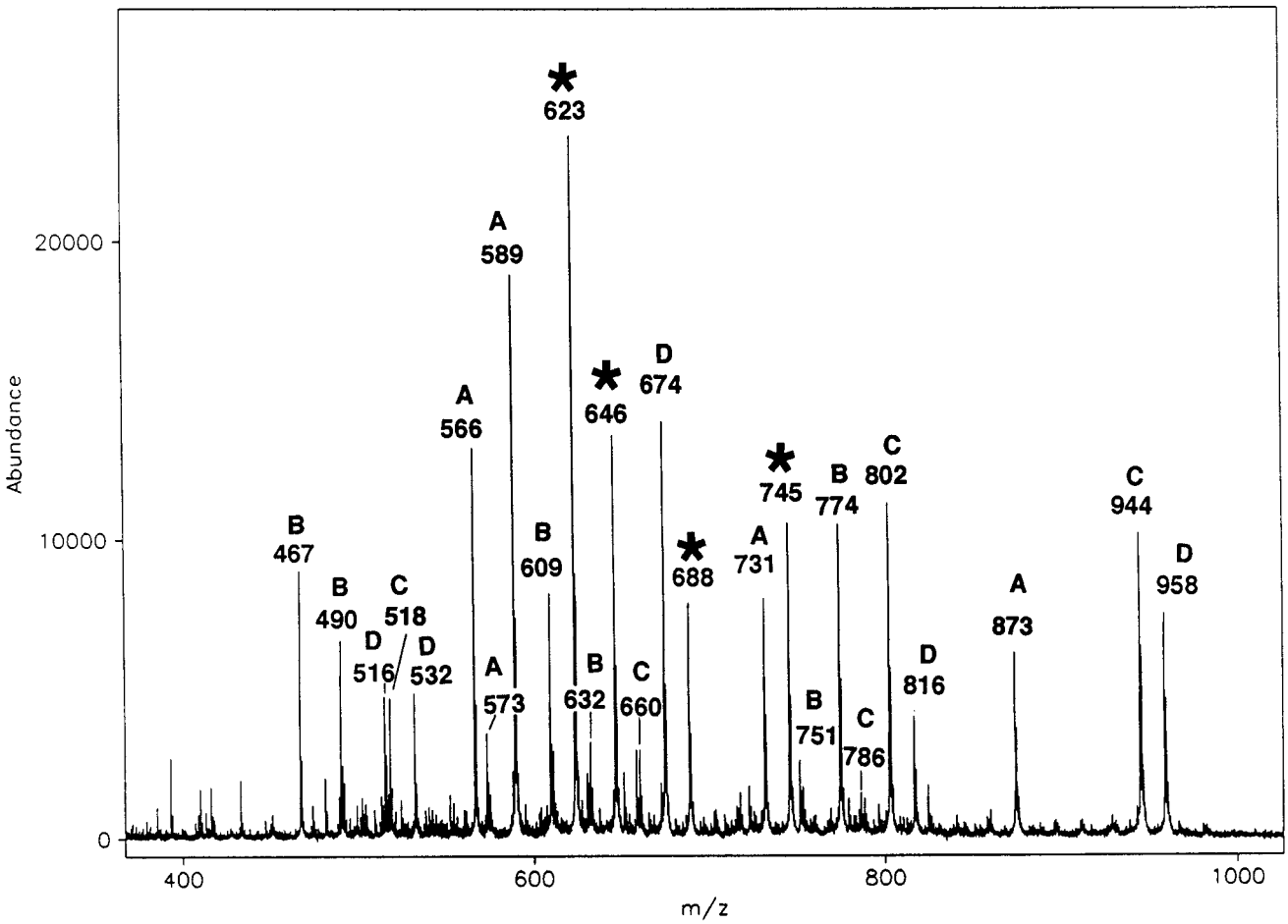


**Fig. 3.** MALDI-sector mass spectrum of crude *Nephilengys cruentata* venom extracts. Among those acylpolyamines, the structures of 4 compounds (* were marked on the mass numbers) were determined by tandem mass spectrometry.

Sample solution (0.1 µl) and saturated α-cyano-4-hydroxycinnamic acid (CHCA) aqueous solution (0.9 µl) was placed on the target and air-dried. An acceleration voltage of 20 kV was used for both linear and reflector mode.

### Tandem Mass Spectrometry

High-energy collision induced dissociation (CID) spectra were measured on JEOL HX110A/HX110A tandem mass spectrometer fitted with a continuous flow FRIT/FAB probe, 6 kV Xe beam FAB gun, and the MS-ADS11 array detector. The CID spectra were recorded with 10 kV acceleration voltage (MS-1, MS-2), and an electrically floated collision cell at 8 kV.

### Nuclear Magnetic Resonance Spectrometry

NMR spectra of individual fractions were taken on a Bruker DMX-500 MHz instrument. The samples were dissolved in deuterated methanol and the aromatic region proton signals were observed for the characterization of lipophilic head moieties.

### Reagents and Supplies

Glycerol for FAB matrix was obtained from Tokyo Kasei (Tokyo, Japan). High performance liquid chromatographic grade acetonitrile and distilled water (Nacalai tesque, Kyoto, Japan) were used for the preparation the samples and mobile phases. All mobile phase solvents contained 0.1% trifluoroacetic acid (TFA) (Nacalai tesque) and were degassed prior to use. 2,5-DHB and CHCA for MALDI matrix were obtained from Wako (Osaka, Japan).

## RESULTS AND DISCUSSION

### Isolation of Acylpolyamines

In the previous studies, we have demonstrated that the combination of µ-column HPLC/MS and high-energy collision MS/MS methods can effectively detect and determine the structures of acylpolyamines stored in the *Nephilengys borbonica* spider venom obtained from Madagascar [Itagaki et al., 1996]. To further explore the potential of mass spectrometric detection and structure determination of acylpolyamines prior to the isolation of those complex compos-

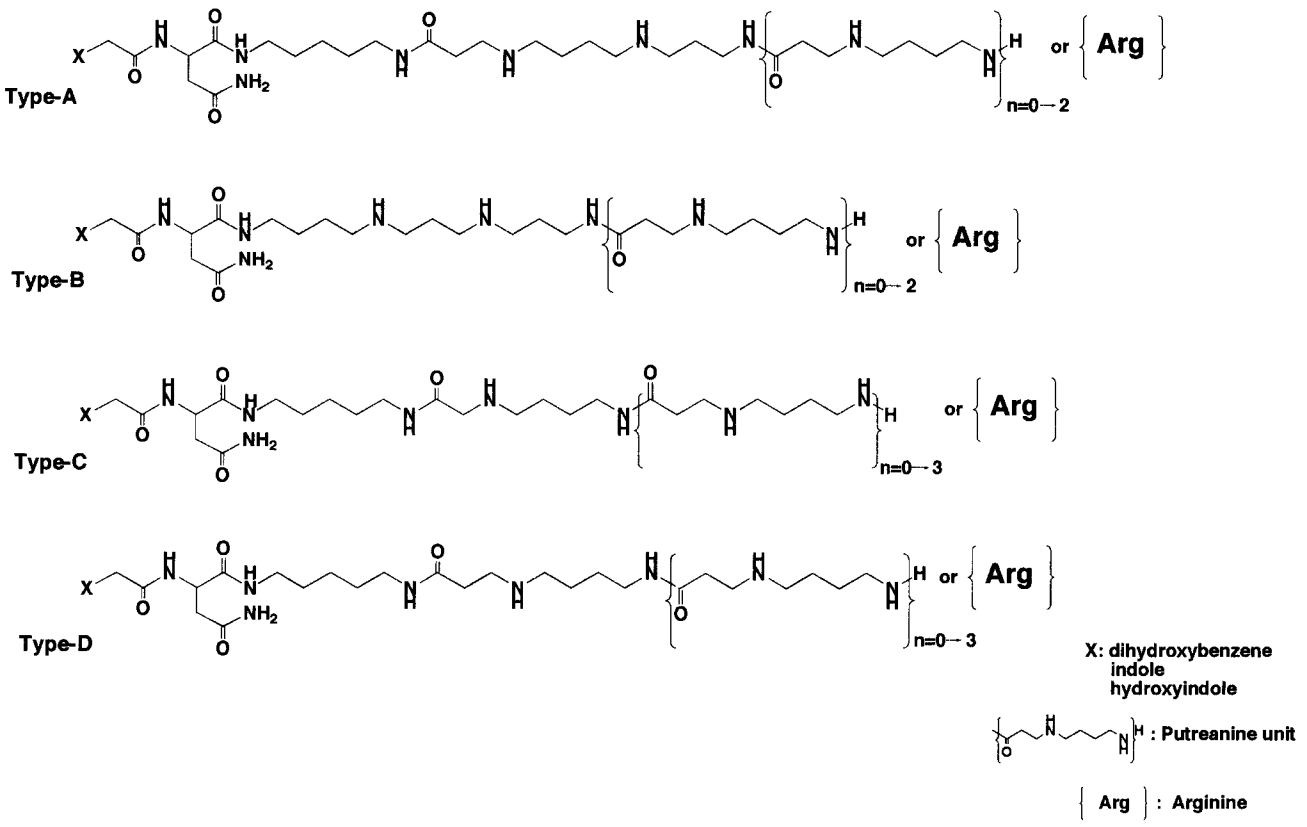


**Fig. 4.** Generalized structures of spider acylpolyamines obtained from *Nephilengys borbonica* and *Nephilengys cruentata* venom extracts.

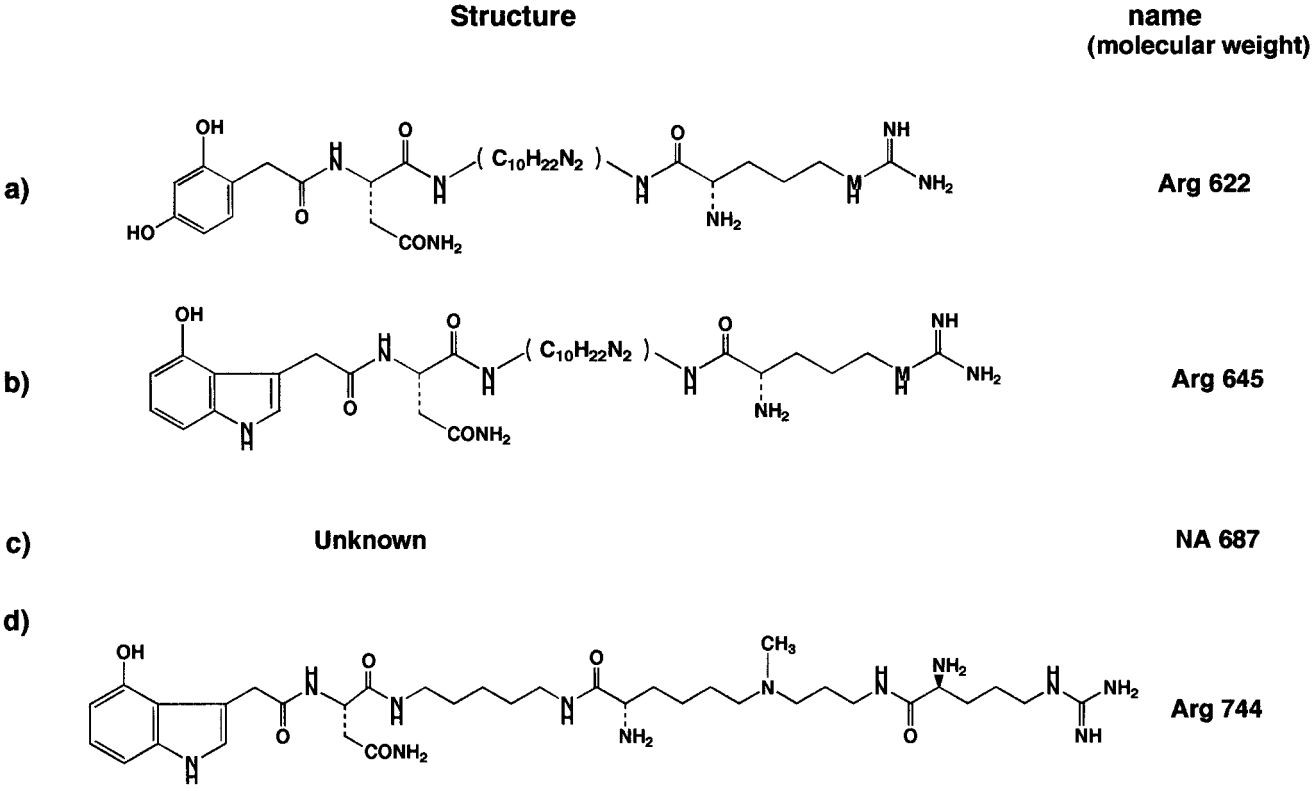


**Fig. 5.** Previously reported acylpolyamines which have the same molecular weight with NPTX-622, -645, -687, and -744.

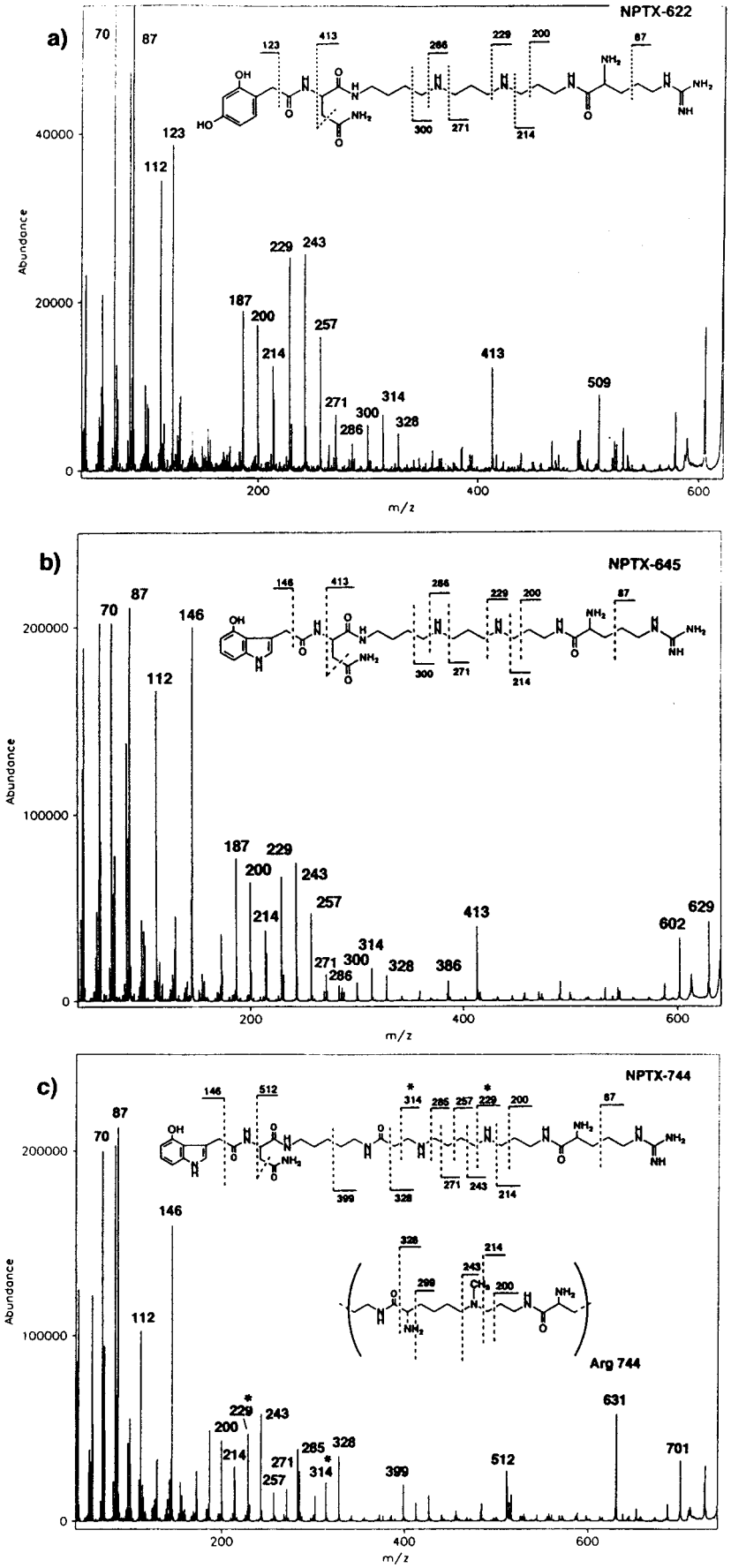


**Fig. 6.** CID spectra of NPTX-622 (**a**), NPTX-645 (**b**), and NPTX-744 (**c**).

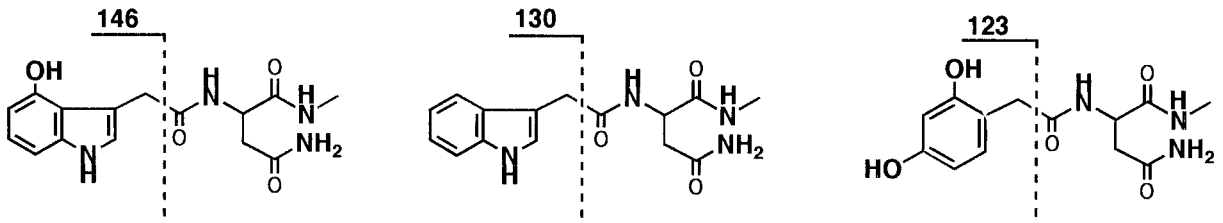


**Fig. 7.** Characteristic product ions to distinguish lipophilic moieties.

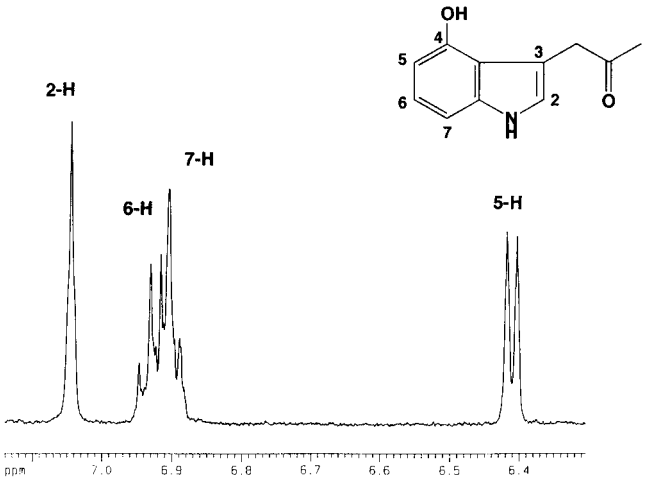


**Fig. 8.** Aromatic region $^1$H NMR signals of hydroxyindole fraction.

ites stored in the spider venoms, the crude venom extracts of *Nephilengys cruentata* collected from Brazil were used. In a set of experiments, we have used μ-column HPLC/MS and MALDI-sector mass spectrometer (Fig. 1) to evaluate the method. The crude venom extracts of *Nephilengys cruentata* was obtained with simple sample preparation procedure and was used for both HPLC/MS and MALDI-sector MS measurements without any further treatment. Protonated molecular ions of venom extracts obtained from those two methods are shown in the two dimensional mass chromatographic display and the MALDI mass spectrum (Figs. 2 and 3), respectively. Twenty-five protonated molecular ions were observed in common from LC/MS and MALDI-sector MS spectra and allowed assignment as the constituents of a single spider venom gland. Although FAB and MALDI mass spectra show different relative peak intensities, exactly the same molecular species were observed by both methods; there are no striking differences using two HPLC/MS and MALDI-sector MS methods. The relative amount of acylpolyamines are not quantitatively reflected in the intensities of the protonated molecular ion peaks; this may be essentially connected to the heterogeneity of MALDI sample preparations and the different hydrophilic/hydrophobic properties of the acylpolyamines. To evaluate the MALDI method, crude mixtures were submitted for measurement by both MALDI-TOF with linear and reflector mode and MALDI-sector MS and showed the same results. Spectra are not shown here but will be discussed elsewhere.

It was confirmed that MALDI mass spectrometry can be used for the identification of each acylpolyamine from complex constituents of a spider venom without purification procedures.

## Structure Determinations of Spider Toxin Acylpolyamines

Among 25 detected protonated molecular ions, the mass numbers of 21 peaks corresponded with the previously determined acylpolyamines which were obtained from *Nephilengys borbonica* venom extracts. By the results of MS/MS spectra and two-dimensional chromatographic distributions, those 21 peaks were assigned to the type A–D structures unambiguously (Fig. 4). The structures of the remaining four compounds, namely, NPTX-622, -645, -687, and -744 (NPTX followed by molecular weight are used for the name of the compounds unless structures were assigned as previously known compounds), which have protonated molecular ions m/z 623, 646, 688, and 745, were subjected to structure determination using high-energy collision induced dissociation (CID) method.

According to the list of polyamine toxins which listed all the currently known and/or detected acylpolyamines [Schäfer et al., 1994], some toxins presenting M.W. 622, 645, 687, and 744 were already known from other species of web spiders and originally named Arg 622, Arg 645, NA 687, and Arg 744 (Fig. 5). However, the structure of NA 687 had been unknown up to now and the three remaining compounds were not precisely characterized. In addition, NPTX-744 apparently showed different mass spectral behavior than Arg 744. Thus, detailed structure determinations were carried out with the four new compounds from the venom of *Nephilengys cruentata.*

### Lipophilic Heads and Polyamine Chain Terminals

The CID spectra of NPTX-622, -645, -687, and -744 (from protonated molecular ions) are shown in Figure 6a–c and Figure 9a. As characteristic peaks at lower mass region, CID spectra of all four compounds showed intense product ions at m/z 70 and 87, which proved the existence of an arginine moiety at the polyamine chain terminal. As we previously reported [Itagaki et al., 1996], the three commonly observed types of lipophilic head can be distinguished by the observation of the product ion at m/z 123, 130, or 146, respectively (Fig. 7). The CID spectra of

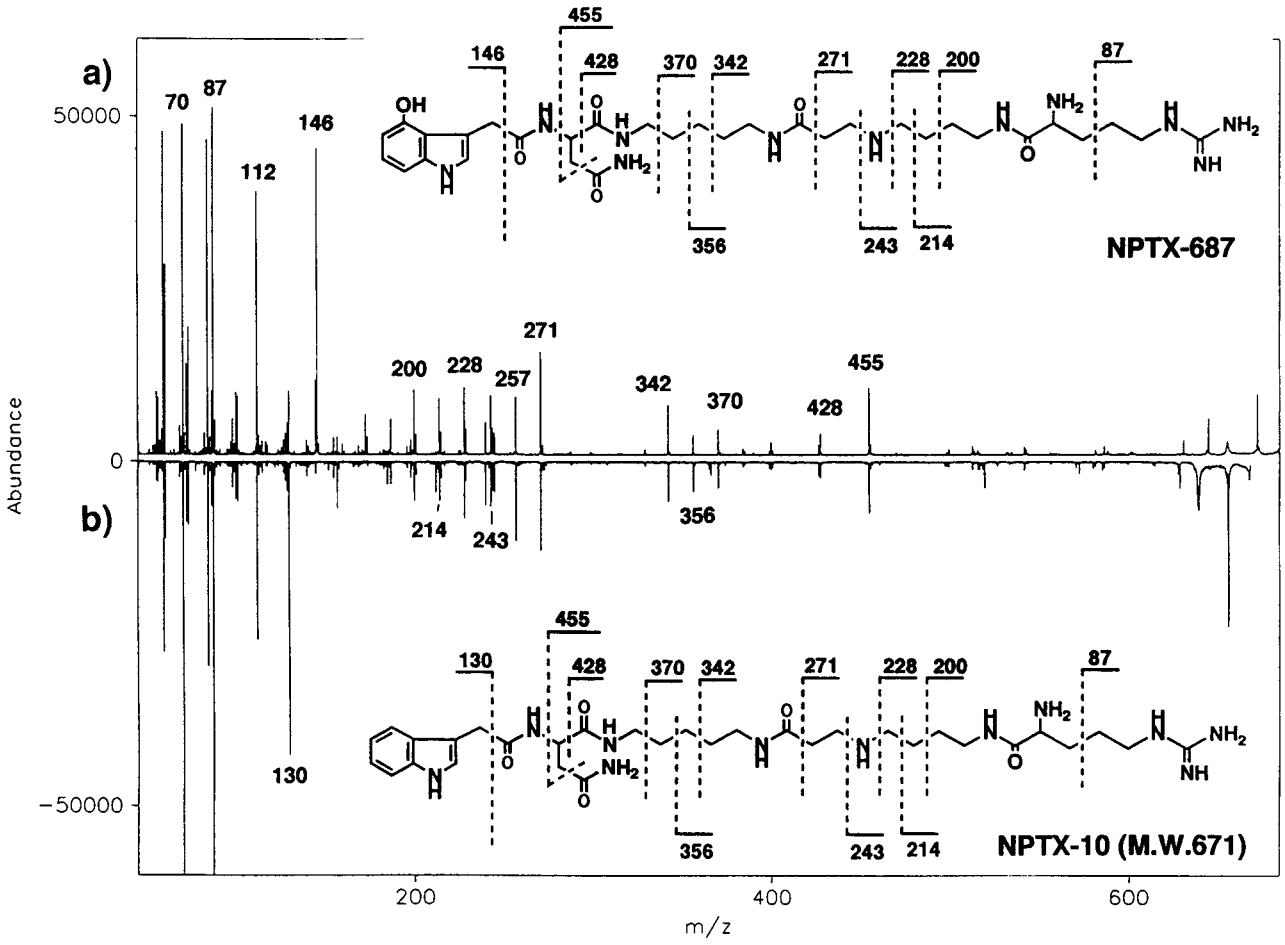


**Fig. 9.** Comparison of CID spectra from **a**) protonated molecular ion of NPTX-687 (m/z688) and **b**) NPTX-10 (synthesized) (m/z672).

NPTX-645, -687, and -744 showed intense product ions at m/z 146 and proved to have hydroxyindole lipophilic head (Fig. 6a,b and 9a,b). On the other hand, NPTX-622 showed an intense peak at m/z 123 instead of m/z 146 indicating a di-hydroxyphenyl acetyl moiety as the lipohilic head.

Though complete separation of complex acylpolyamine is difficult, the polyamines were separated into three fractions, each one containing either dihydroxyphenyl-, hydroxyindole-, or indole-moiety by reverse-phase HPLC, following the previously reported experimental procedure. Among these lipophilic moieties, 2,4-dihydroxybenzene- and indole- have been commonly found from various acylpolyamines. The location of the hydroxyl group in the hydroxyindole moiety was previously proposed at position-6 [Toki et al., 1988]; however, we later reexamined location by using NMR measurements to assign the hydroxyl group into position-4 [Shinada et al., 1996]. The fraction from the venom of *Nephilengys cruentata* with a hydroxyindole head was submitted to proton NMR measurements to confirm this new location. The signals observed in the aromatic region (Fig. 8) were consistent with the presence of 4-hydroxyindole, i.e., doublet at δ 6.40, 6.41 (5-H), complex multiplet at δ 6.90 to 6.93 (6 and 7-H), and singlet at δ 7.04 (2-H). From NMR data, the hydroxyl group position of all hydroxyindole acetyl polyamines obtained from *Nephilengys cruentata* were confirmed to have a 4-hydroxyindole acetyl group.

Remaining structures (polyamine chain connectivity) can also be determined by the analysis of CID spectra of protonated molecular ions. Unlike the previously determined acylpolyamines obtained from *Nephilengys borbonica* spider venom or coexisting 21 acylpolyamines of *Nephilengys cruentata* which have putreanine moiety at polyamine chain terminals, it does not require addition of alkaline salts to form charge localized sodiated molecular ions. Because of the existence of basic amino acid arginine as a charge localized moiety, the CID spectra of those four compounds showed mainly product ions which retained the positive charge at the arginine moiety. Therefore, high-energy CID spectra from protonated molecular ions afforded the facile interpretation of acylpolyamine connectivity.

### Structures of NPTX-622 and NPTX-645

As we previously reported, 23 Da mass difference between NPTX-622 and -645 suggested that the structure

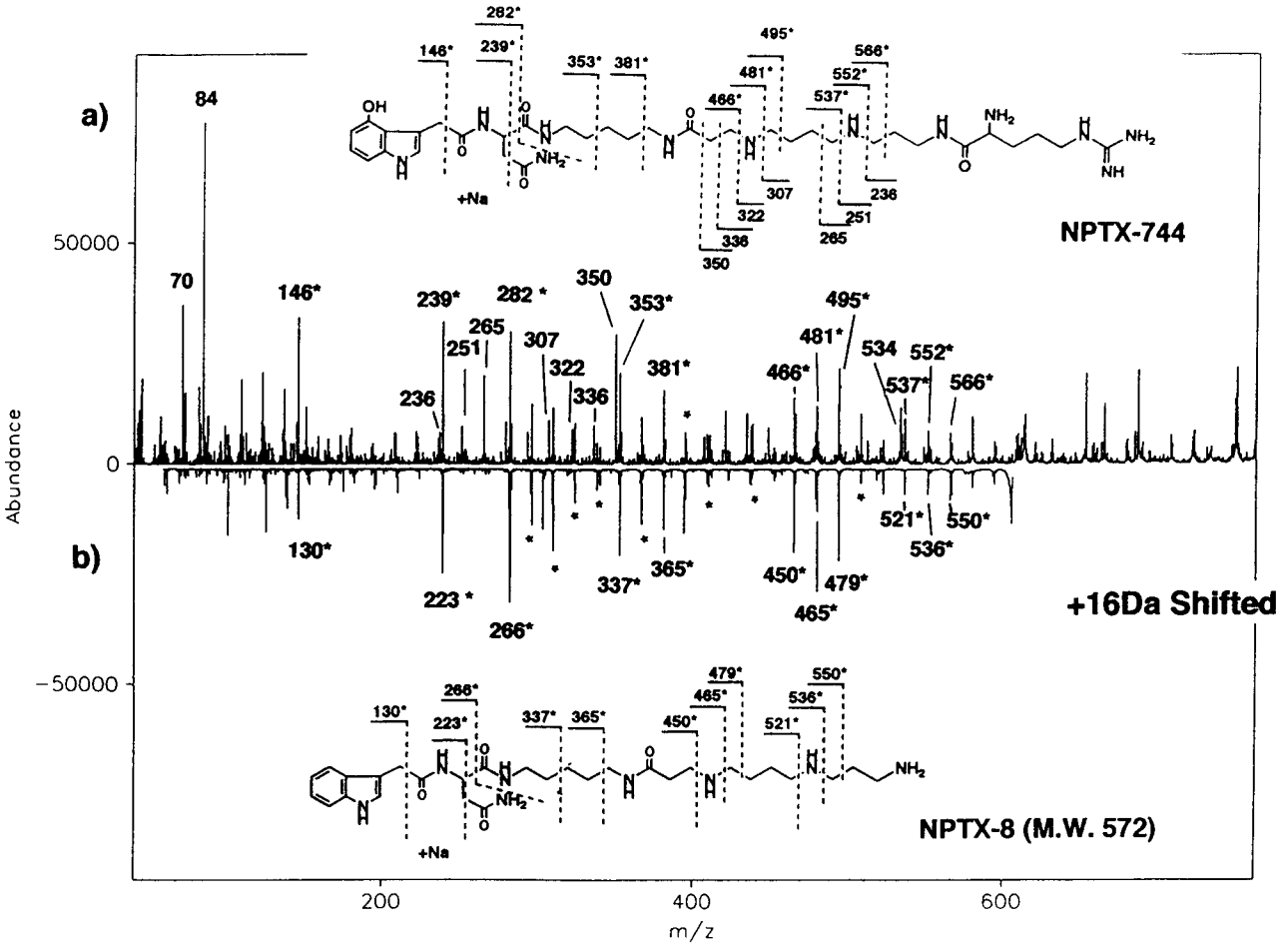


**Fig. 10.** Comparison of CID spectra from sodiated molecular ion of NPTX-744 (**a**) and NPTX-8 (synthesized) (m/z 595) (**b**). The latter spectrum has been plotted after +16Da shift. *: Charge is retained on a lipophilic side.

could be only the difference of dihydroxyphenyl and 4-hydroxyindole moieties having the same polyamine chain. Due to the nature of high-energy collisions, the compounds which have the same polyamine chain should show the same fragmentation pathway with similar product ion intensities [Fujita et al., 1995]. Commonly observed arginine moiety charge retained product ions at m/z 187, 200, 214, 229, 243, 257, 271, 286, 300, 314, 328, and 413 on both NPTX-622 and -645 CID spectra (Fig. 6a,b), allowed us to infer a three-segmented polyamine chain 4-3-3 ($C_4$-$C_3$-$C_3$ unit) type-B structure having an arginine at chain terminal instead of putreanine. From the observation of a 15 Da mass difference between m/z (214–229) and m/z (271–286), the location of nitrogen atoms and the connectivity of methylene numbers were determined. Previously, Arg 622 and 645, which have the same molecular weight as NPTX-622 and -645, respectively, were obtained from orb-web spiders *Argiope trifasciata* and *Argiope florida* as minor components of argiotoxin 636 and 659, having the structures of 2,4-dihydroxyphenyl acetyl- and hydroxyindole acetyl-asparagine linked through a C11-tetra-amine to N-terminal arginine (Fig. 5a,b) [Budd et al., 1988]. Because of a limited amount of sample, they could not determine the absolute structures of polyamine moieties. Even though we have detected the same molecular weight compound from different species, Arg 622 and 645 could have the same structure as NPTX-622 and -645; but until this is confirmed, NPTX-622 and -645 will be used for these compounds.

### Structure of NPTX-687

The structure of NPTX-687 was confirmed by the comparison of the CID spectrum of synthesized NPTX-10 (Fig. 9a,b). All product ions carrying the charge at arginine moieties appeared at the same mass numbers m/z 455, 428, 370, 356, 342, 271, 257, 243, 228, 214, and 200 with similar intensities on both spectra, permitting to classify it as a type-D like structure having an arginine at the chain tail instead of putreanine. Mass spectral difference at m/z 146 and 130 together with NMR results also support that NPTX-687 has a 4-hydroxyindole acetyl moiety as lipo-

philic head. NPTX-687 was also found from the venom of Joro spider *Nephila clavata* during the confirmation of hydroxyl group position.

### Structure of NPTX-744

Product ions observed at m/z 200, 214, 229, 243, 257, 271, 285, 314, and 328 indicate that NPTX-744 has part of its polyamine chain containing a 4-3 methylene connectivity. All sodium attached acyl or arginine side charge retaining product ions support the proposed NPTX-744 and allows classification as a type A structure, with an arginine at the terminal portion of chain instead of putreanine (Fig. 6c). As polyamine chain, Arg 744 has primary amine and methyl groups as side chains. If this is the structure of NPTX-744, the CID spectrum should not show product ions such as m/z 229 and 314 (product ions are shown with an asterisk in Fig. 6c) because high-energy CID dominate the single cleavage process and is not likely to produce adjacent C-C and C-N bond-cleaved product ion. A linear polyamine chain structure was confirmed by the comparison of sodiated molecular ions of NPTX-744 and already known NPTX-8 (synthetic), which was supposed to have the same polyamine chain. The CID spectrum of sodiated NPTX-744 was displayed together with +16 Da (oxygen atom difference) shifted NPTX-8 (Fig. 10a,b). CID spectra became rather complicated because of the formation of both acyl group and arginine side charge retained product ions but the differentiation of those product ions can be easily done by 16 Da shifted spectrum display (* were marked for acyl group charge retained ions). The ion species of m/z 466, 481, 495, 537, 552, and 566 were corresponded with 16 Da less product ions of NPTX-8; m/z 450, 465, 479, 521, 536, and 550, respectively.

## CONCLUSIONS

The MALDI-sector MS was successfully applied for the detection of acylpolyamines from crude spider venom extracts. Using venom from one *Nephilengys curuentata* spider, 25 toxins were identified as acylpolyamines. Among those, 21 compounds were characterized by HPLC retention time and comparison with the CID spectrum of previously determined standard samples. The structures of four previously unknown compounds were characterized using high energy CID spectra of protonated molecular ions as arginine containing acylpolyamines. This method shall be applicable to finding out other compounds stored not only in spider venom glands but also in other animal venoms.

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